Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish

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ABSTRACT

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Aims: To evaluate the intraspecific diversity of the fish pathogen *Flavobacterium columnare*

Methods and Results: Genetic variability among *Fl. columnare* isolates was characterized using restriction fragment length polymorphism analysis of the 16S rDNA gene, intergenic spacer region (ISR) sequencing, and amplified fragment length polymorphism (AFLP®) fingerprinting. Thirty *Fl. columnare* cultures isolated from different fish species and geographical origins as well as reference strains were included in the study. Fifteen isolates belonged to genomovar I while eleven were ascribed to genomovar II. Analysis of the ISR sequence confirmed the genetic differences between both genomovars but revealed a higher diversity among genomovar I isolates. The maximum resolution was provided by AFLP® fingerprinting, as up to 22 AFLP profiles could be defined within the species.

Conclusions: We confirmed the division of *Fl. columnare* isolates from cultured fish into different genogroups. We showed that both genomovars I and II are present in channel catfish from the US. We described a unique genetic group represented by four *Fl. columnare* isolates from tilapia in Brazil which appears to be related to both genomovars. We were able to further subdivide the species by analysing the ISR. Finally, the use of AFLP® allowed us to fingerprint the species at clone level without losing the higher genetic hierarchy of genomovar division.

Significance and Impact of the Study: This paper reports on an extensive assessment of the use of molecular tools for the study of the epidemiology of the fish pathogen *Fl. columnare*.

Keywords: amplified fragment length polymorphism, catfish diseases, *Flavobacterium columnare*, genomovars, genotyping, intergenic spacer region.

INTRODUCTION

Flavobacterium columnare is the causal agent of columnaris disease, one of the most important bacterial diseases of freshwater fish species. This bacterium is distributed world wide in aquatic environments, affecting wild and cultured fish as well as ornamental fish in aquaria (Decostere *et al.* 1998; Austin and Austin 1999). Members of the ictalurid family, especially channel catfish (*Ictalurus punctatus* Rafinesque) are particularly susceptible to columnaris (Meyer

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1970). The disease generally begins as an external infection on fins, body surface, or gills. Fin lesions can become necrotic, while skin lesions have yellowish mucoid material. Lesions can develop exclusively on the gills, which usually result in subacute disease and mortality. In some cases columnaris becomes systemic with little or no visible pathological signs. *Flavobacterium columnare* infections can be chronic and cause lingering, gradually accelerating mortality in channel catfish but more often, the disease appears suddenly and accelerates to subacute mortality within a few days (Austin and Austin 1999).

From 1987 to 1989, columnaris was the most frequently reported infectious disease in the US catfish industry

accounting for 58% of all bacterial cases (Thune *et al.* 1993). Currently, *Fl. columnare* is considered the second most important bacterial pathogen in commercial culture of channel catfish in the South-eastern United States, second only to *Edwardsiella ictaluri*. Mortality rates of catfish populations in ponds can reach 50–60% and can be as high as 90% in tank-held catfish fingerlings (National Animal Health Monitoring System (NAHMS) 1997).

Although columnaris negatively impacts aquaculture production around the world, bibliographical references to this species are mostly related to its taxonomical status not to its pathogenesis or epidemiology. After undergoing several taxonomic reclassifications and name changes, Bernardet *et al.* (1996) redefined the Flavobacteriaceae family and finally renamed the bacterium as *Flavobacterium columnare*. Subspecies characterization of *Fl. columnare* has been difficult in the past as most isolates are phenotypically homogeneous, which constitutes a limitation for strain definition. However, it has been reported that *Fl. columnare* presents a wide genetic heterogeneity within the species. Triyanto and Wakabayashi (1999) described three different genomovars among the species based on the analysis of the 16S rDNA gene Restriction Fragment Length Polymorphisms (16S rDNA-RFLP). The authors demonstrated that, although all *Fl. columnare* isolates were phenotypically identical, the 16S rDNA sequence provided enough variability to defined three genomovars within the species. This data was supported by DNA:DNA hybridization experiments. Michel *et al.* (2002) used the same methodology to characterize *Fl. columnare* cultures isolated from neon tetras (*Paracheirodon innesi* Genri). According to these authors, genomovar II or Asian type strains might have been brought to Europe through ornamental fish imports.

The purpose of the present study was to explore in depth the intraspecific diversity of *Fl. columnare* isolates from cultured fish. To achieve this objective we applied three genotyping methods to *Fl. columnare* cultures isolated from different sources. First, we used the Triyanto and Wakabayashi 16S rDNA-RFLP strategy to define genomovars. Secondly, we analysed the variability of the Intergenic Spacer Region (ISR) that has been successfully used in other bacterial species for strain characterization (Gurtler and Barrie 1995; Zavaleta *et al.* 1996). Finally, we used a more complex but highly discriminative typing technique, Amplified Fragment Length Polymorphism (AFLP®) (Vos *et al.* 1995) to reveal the polyclonal nature of *Fl. columnare*.

MATERIALS AND METHODS

Bacterial isolates

Thirty *Fl. columnare* isolates from different sources, including reference strains, were used in this study (Table 1). All environmental strains have been fully characterized and identified as *Fl. columnare* based on standard biochemical methods (Bernardet *et al.* 1996). Stock suspensions of all isolates were stored in 10% glycerol at −70°C. After thawing, the bacteria were grown in Shieh broth (Shieh 1980) for 24 h at 25°C with gentle shaking.

16S rDNA-RFLP analysis

Amplification of the 16S rDNA gene was carried out according to Triyanto and Wakabayashi (1999). Universal primers 20F (5′-AGAGTTTGATC(AC)TGGCTCAG-3′) (position 8–27 of *Escherichia coli* numbering) and 1500R (5′-CGATCCTACTTGCGTAG-3′) (position 1510–1492 *E. coli* numbering) were used to amplify the 16S rDNA gene. Amplified products were digested using the restriction endonucleases *Hae*III, *Hha*I, *Msp*I, *Hin*fI, and *Rsa*I. Following digestion, restriction fragments were electrophoresed in a 2% agarose gel, stained with ethidium bromide, and photographed under u.v. light. Gel images were converted, normalized, and restriction patterns were analysed using the software package BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium).

ISR sequencing analysis

Universal primers 16S-14F (5′-CTTGTACACACCGCCCGTC-3′, position 1389–1407 *E. coli* numbering) and 23S-1R (5′-GGGTTTCCCCATTCGGAAATC-3′, position 124–110, *E. coli* numbering) against highly conserved regions were used to amplify the 16S-23S rDNA ISR (Zavaleta *et al.* 1996). One single colony was resuspended in sterile water, boiled for 5 min, cooled on ice, and centrifuged briefly. Five microlitres of supernatant were used as template DNA for PCR amplification. PCR conditions were as described previously by Zavaleta *et al.* (1996). Amplified products were cleaned up using the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation, Indianapolis, IN, USA) and sequenced by the Auburn University Genomics and Sequencing laboratory. Calculation of similarity values and cluster analysis were done using the BioNumerics software taking into account the homologous nucleotide positions after discarding all unknown bases and gaps. A dendrogram was constructed, using the same software package, employing the neighbour-joining method. Tree robustness was defined by a bootstrap analysis of 1000 replicates.

AFLP® analysis

AFLP® fingerprints were determined as previously described by Arias *et al.* (1997). Briefly, total DNA was extracted using the DNeasy Tissue kit (Qiagen, Valencia,

Table 1 *Flavobacterium columnare* strains used in the study

Strain	Source	Geographic origin	Date	Genomovar	ISR	AFLP
ATCC 23463[T]	Chinook salmon	Washington, USA	Unknown	I	–	17
ATCC 49512	Brown trout	France	1987	I	–	17
ALG-03-057	Channel catfish	Alabama, USA	2003	I	–	16
ALG-03-063	Channel catfish	Alabama, USA	2003	I	–	15
ALG-03-069	Channel catfish	Alabama, USA	2003	I	A	22
27	Channel catfish	Alabama, USA	2002	I	–	12
HS	Channel catfish	Alabama, USA	Unknown	I	–	19
GZ	Channel catfish	Alabama, USA	2000	I	A	18
ARS-1	Channel catfish	Alabama, USA	1996	I	A	20
BioMed	Channel catfish	Alabama, USA	1996	I	A	20
MS-02-463	Channel catfish	Mississippi, USA	2002	I	–	14
MS-02-465	Channel catfish	Mississippi, USA	2002	I	–	11
MS-02-467	Channel catfish	Mississippi, USA	2002	I	–	11
GA-02-14	Rainbow trout	Georgia, USA	2002	I	A	21
IR	Common carp	Israel	Unknown	I	–	13
ALG-00-513	Channel catfish	Alabama, USA	2000	II	C	1
ALG-00-515	Channel catfish	Alabama, USA	2000	II	C	3
ALG-00-521	Channel catfish	Alabama, USA	2000	II	D	9
ALG-00-522	Channel catfish	Alabama, USA	2000	II	D	6
ALG-00-527	Channel catfish	Alabama, USA	2000	II	D	5
ALG-00-530	Channel catfish	Alabama, USA	2000	II	C	1
ALG-02-036	Largemouth bass	Alabama, USA	2002	II	C	4
PT-14-00-151	Channel catfish	Mississippi, USA	2000	II	C	2
MS-02-475	Channel catfish	Mississippi, USA	2002	II	D	7
LSU	Channel catfish	Louisiana, USA	1999	II	C	1
MO-02-23	Largemouth bass	Missouri, USA	2002	II	C	1
BZ-1-02	Nile tilapia	Brazil	2002	ND	B	8
BZ-2-02	Nile tilapia	Brazil	2002	ND	B	10
BZ-4-02	Nile tilapia	Brazil	2002	ND	B	8
BZ-5-02	Nile tilapia	Brazil	2002	ND	B	8

[T]Type strain.

CA, USA) following manufacturer's instructions. One hundred nanogram of total DNA was digested with 10 units of *Hin*dIII and *Taq*I (Promega, Madison, WI, USA) in a final volume of 30 μl. Following digestions, adapters were added to a final concentration of 0·04 and 0·4 μM for *Hin*dIII- and *Taq*I-adapters respectively, and ligated to the restriction fragments using T4 DNA ligase (Promega). AFLP® reactions employed two specific primers, oligonucleotide T000 (5′-CGATGAGTCCTGACCGAA-3′) corresponding to the *Taq*I-ends, and H00A corresponding to the *Hin*dIII-ends (5′-GACTGCGTACCAGCTT<u>A</u>-3′, selective base at the 3′-end is underlined). *Hin*dIII primer H00A was labelled with an IR700 fluorochrome from LI-COR (Lincoln, NE, USA). PCR conditions are described elsewhere (Arias *et al.* 1997). The PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions. AFLP® images were processed with BioNumerics. Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Pearson product-moment correlation coefficient (r). Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA).

RESULTS

16S rDNA-RFLP analysis

Nucleotide sequences of approx. 1500 bp were amplified from the 16S rDNA of 26 *Fl. columnare* strains. After restriction of the amplified product with *Hae*III, we found two different genetic profiles corresponding to genomovars I and II (Fig. 1). Fifteen isolates displayed the typical pattern of genomovar I (Table 1), while 11 isolates belonged to genomovar II. All salmonid isolates, American (ATCC 23463[T], and GA-02-14) and European (ATCC 49512), belonged to genomovar I. Among the warm water fish
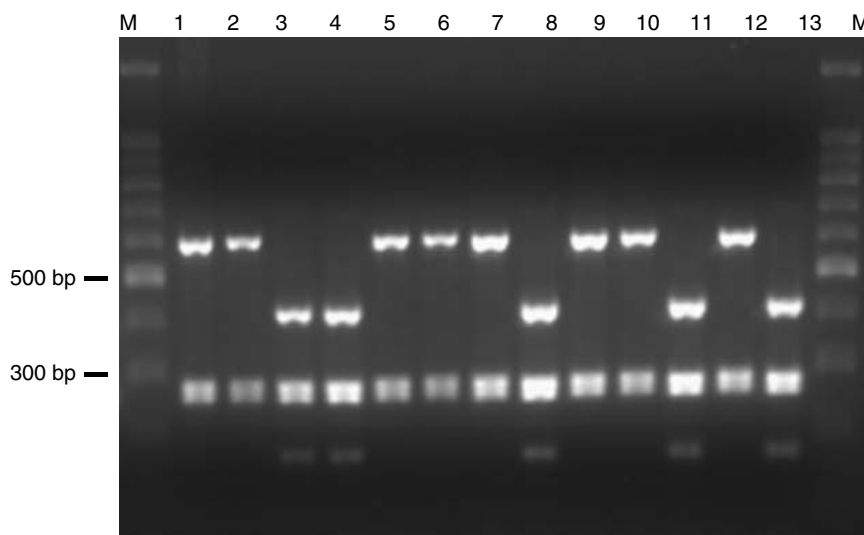
Fig. 1 Restriction profiles of 16S rDNA from 13 *Fl. columnare* strains digested with *Hae*III. Lanes 3 (ATCC 23463), 4 (ALG-03-69), 8 (IR), 11 (ARS-1), and 13 (MS-02-467) show the typical genomovar I profile. Lanes 1 (ALG-00-513), 2 (MO-02-23), 5 (LSU), 6 (ALG-00-521), 7 (ALG-00-522), 9 (ALG-02-036), 10 (MS 475), and 12 (PT-14-00-151) display genomovar II profiles. Lanes M, 100 bp molecular weight marker

isolates, 57% exhibited the typical genomovar I pattern, and 43% were ascribed to genomovar II. Out of 20 channel catfish isolates, 11 corresponded to genomovar I and nine to genomovar II. The two largemouth bass (*Micropterus salmoides*) isolates shared a genomovar II profile while the only carp (*Cyprinus carpio*) isolate belonged to genomovar I. No correlation between geographical origin and genomovars was found in our study. Genomovars I and II appeared to be equally represented among the South-eastern states. None of the isolates belonged to genomovar III.

We were not able to amplify the 16S rDNA from *Fl. columnare* isolates from Brazil (BZ-1-02, BZ-2-02, BZ-4-02, and BZ-5-02) using the universal primers described by Weisburg *et al.* (1991). Therefore, adscription to genomovar was not possible for these four tilapia (*Oreochromis niloticus*) isolates.

ISR sequencing analysis

Amplification with universal primers 16S-14F and 23S-1R yielded a single amplified product of approx. 600 bp, indicating that the ISR size is similar in all ribosomic operons along the chromosome, as well as common to all *Fl. columnare* strains analysed. Brazilian isolates represented an exception as they showed two distinct ISR amplified bands of 400 and 600 bp. Only the 600 bp fragment was used for sequence comparison. Direct sequencing of the ISR amplified products were obtained and deposited in GenBank. Partial sequence lengths ranged from 505 to 600 bp with an average C + G content of 33%. After multiple alignment, a distance matrix was generated and a neighbour-joining

dendrogram was constructed based on evolutionary distance values previously calculated (Fig. 2). The phylogenetic tree confirmed the split of the species into two different groups that correlated to genomovars I and II. All members of genomovar II clustered at 67% similarity. Genomovar I displayed a higher degree of intrinsic variability with a minimum similarity of 59%. Interestingly, Brazilian isolates formed a distinct yet homologous group in between genomovars I and II. Based on tree topology and robust bootstrap values (higher than 80%), four main ISR groups were defined (Table 1). Group A clustered genomovar I isolates from Alabama and Georgia. Cluster B grouped all Brazilian isolates. Genomovar II isolates from Alabama, Louisiana, Mississippi, and Missouri, were split between groups C and D.

AFLP®

Figure 3 shows the results of the cluster analysis of the AFLP® patterns. It is noteworthy that this technique yielded isolate-specific patterns of 35 to 50 distinct bands ranging from 50 to 550 bp. At 85% similarity (cut-off value for our AFLP® analysis), 22 distinct AFLP® profiles were defined (Fig. 3). AFLP® profile 1 was the most common pattern shared by four *Fl. columnare* isolates. Interestingly, these isolates came from different geographical origins and fish species. In fact, the isolates ALG-00-530, ALG-00-513 (channel catfish, Alabama), MO-02-23 (Missouri, largemouth bass), and LSU (Louisiana, channel catfish) presented almost identical AFLP® profiles at more than 90% similarity. Three of the Brazil cultures isolated from tilapia
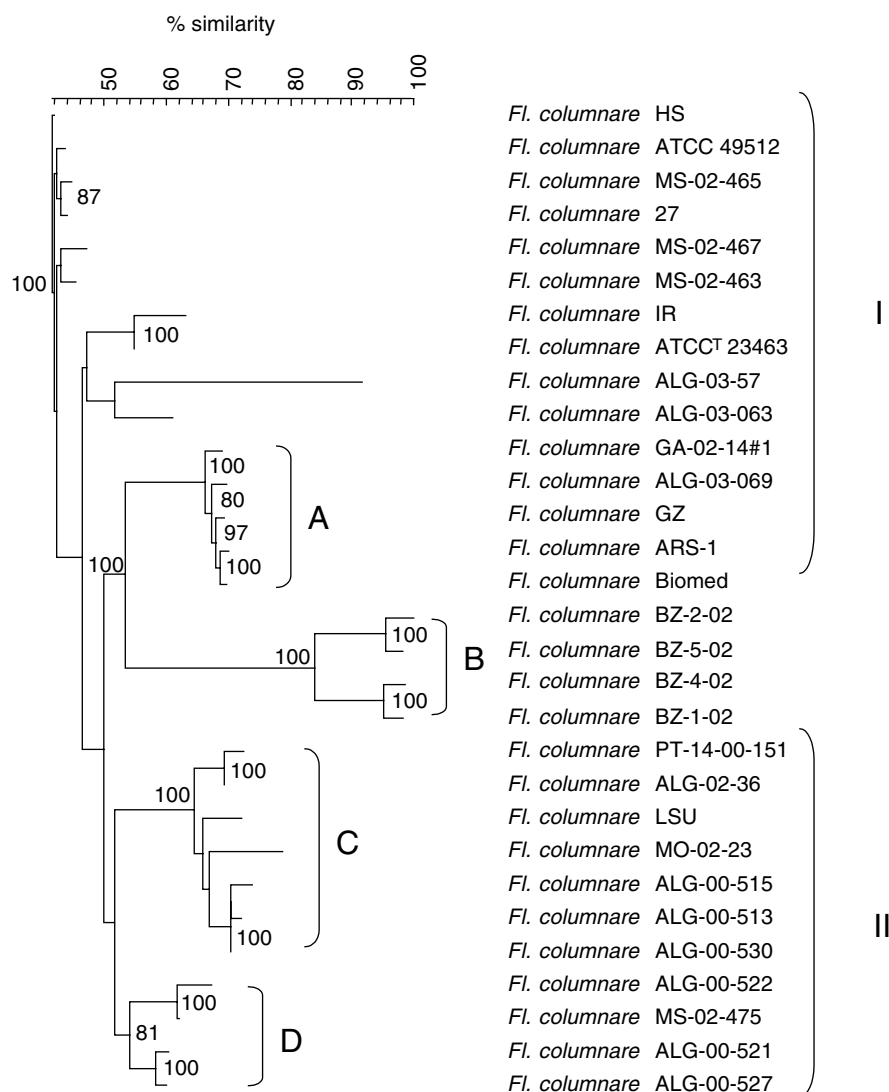
Fig. 2 Phylogenetic tree based on ISR sequences of all 30 *Fl. columnare* isolates analysed in the study. Numbers depict significant bootstrap values (>79%) obtained for a bootstrap sampling of 1000. ISR groups A–D, as well as genomovars I and II, are indicated

also formed a tight group, profile 8. However, they were considerably similar (70%) to a channel catfish isolate from Alabama (ALG-00-521). It is also remarkable that the two reference strains, both isolated from salmonids although from distant geographical locations, displayed an identical AFLP® pattern (profile 17). The remaining two isolates sharing a common AFLP® pattern (20) were both isolated from channel catfish in Alabama.

Good agreement between AFLP® fingerprinting and genomovar ascription was observed. *Flavobacterium column-are* genomovar II isolates showed AFLP® profiles 1 to 7. Genomovar I isolates corresponded to AFLP® patterns 11 to 22. Brazilian isolates clustered in the middle of both genomovars, showing AFLP profiles 8 and 10. Isolate

ALG-00-521 represented the only exception to AFLP® fingerprinting and genomovar agreement. This genomovar II isolate clustered in between the Brazilian isolates sharing a higher similarity with them than to the rest of the genomovar II isolates.

DISCUSSION

The first attempt to discriminate between *Fl. columnare* isolates was made by Anacker and Ordal (1959). These authors divided the species into four different serotypes and one miscellaneous group. Song *et al.* (1988) found three types of colony morphology among the species. Several authors have reported slight phenotypic variations within
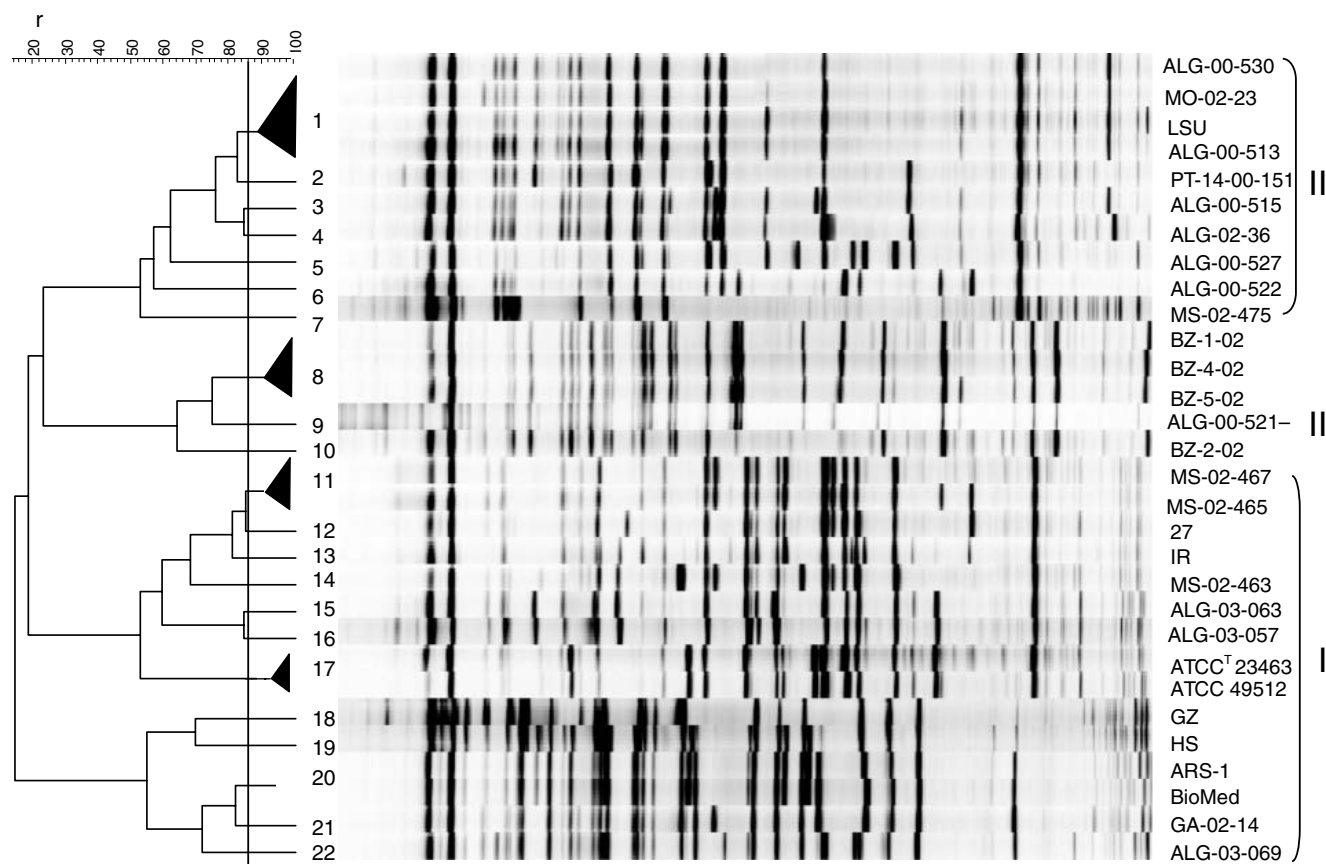
Fig. 3 AFLP patterns of *Fl. columnare*. The dendrogram was derived by UPGMA cluster analysis of the AFLP profiles of 30 *Fl. columnare* strains. The tracks show the processed band patterns after conversion, normalization, and background subtraction. Levels of linkage are expressed as the Person product-moment similarity coefficient. Numbers correspond to AFLP profiles 1–22. Isolate ascription to genomovars I and II is also noted

Fl. columnare such as ability to grow at 15 and 37°C (Bernardet 1989) and nitrate reduction (Shamsudin and Plumb 1996). In contrast to a high phenotypic and physiological homogeneity, *Fl. columnare* presents a wide genetic diversity. DNA relatedness studies between *Fl. columnare* strains revealed homologies as low as 78% (Bernardet and Grimont 1989). Toyama *et al.* (1996) showed an existing intra-species variation among strains of *Fl. columnare* based on 16S rDNA sequences. This work set up the basis for the Triyanto and Wakabayashi (1999) study that led to the description of three genomovars within this species. Genomovars were defined based on RFLP analysis of the 16S rDNA gene but were also confirmed by DNA:DNA hybridization. DNA relatedness between strains belonging to the same genomovar was higher (ranging from 83 to 100% homology) than between strains from other genomovars (homologies lower than 69%).

In this study, we analysed the intra-species genetic diversity of *Fl. columnare* isolates from the main aquaculture areas in the South-eastern US and we compared them with isolates from distant geographical locations as well as reference strains. We found that, based on the RFLP-16S rDNA analysis, genomovars I and II were equally represented in South-eastern US. Our data suggest that channel catfish is susceptible to both genomovars, without any bias based on geographical location or date. It also seems clear that genomovar I isolates can infect cold and warm water fish, while genomovar II was only recovered from warm water species. Genomovar II has been referred to as *Fl. columnare* Asian genomovar in the literature (Michel *et al.* 2002). However, our study showed that this genomovar is commonly present in the US. Brazilian isolates from tilapia constituted a unique group as we were unable to amplify their 16S rDNA. The fact that universal primers against highly conserved regions in the 16S ribosomic genes failed to yield the expected amplified product denotes the singularity of these isolates.

To further investigate the genetic variability of our isolates, we analysed the ISR sequence located between the 16S and the 23S rDNA genes. This sequence is known

to be under less evolutionary pressure and, therefore, to provide greater genetic variation than rDNA coding regions such as the 16S rDNA gene (Zavaleta *et al.* 1996). The phylogenetic tree based on ISR sequences revealed a higher diversity within genomovar I isolates, with some isolates sharing a similarity lower than 50%. Only one clear ISR group could be defined within genomovar I isolates. By contrast, genomovar II isolates appeared as a more homogeneous group clustering at 50% similarity and showing two perfectly defined ISR groups. Brazilian isolates seemed to be more related to genomovar I than to genomovar II.

Finally, by using AFLP® we were able to fingerprint the entire genome of *Fl. columnare*. As with ISR, AFLP® confirmed three main groups within our isolates: genomovars I and II and the Brazilian isolates cluster. However, AFLP® also showed the polyclonal nature of this species as isolates from distant geographical locations showed surprisingly similar AFLP® profiles. This was the case for AFLP® profile 1 shared by isolates from Alabama, Louisiana, and Missouri. Also both ATCC reference strains used in the study, one European and one American isolate, showed the same AFLP® profile. Another example was provided by the Israeli isolate (IR), which clustered with several Alabama and Mississippi isolates. Based on our AFLP® data, different clonal lines could be defined within the species. Moreover, it appears that some of them are distributed worldwide. No association between fish species and *Fl. columnare* clones could be inferred at this point.

In general we found a good agreement between all three typing methods used in this study. Our data strengthen the division of *Fl. columnare* into genomovars and we consider RFLP 16S rDNA analysis an easy way for genomovar ascription. However, genomovar ascription was not possible in four *Fl. columnare* isolates as amplification of the 16S gene using universal primers failed. These four isolates formed a different group by all three genotyping methods used and might constitute a new genomovar within the species (genomovar IV). However, this hypothesis will need to be confirmed by sequencing the 16S RNA gene from these isolates. A primer-walking sequencing strategy, starting from the ISR, might be needed to overcome the inability of the 16S universal primers to yield an amplified product. Additional universal primers 16S-14R (5′-CTTGTACA-CACCGCCCGTC-3′ and E1541R 5′-AGGAGGT GATCCAACCGCA-3 complementary to positions 1389–1407 and 1520–1539 in *E. coli*, were also tested with these isolates in combination with forward primer 20F. None of them produced an amplified product. As we succeeded in amplifying the ISR sequence using primer 16S-14F as one of the universal primers, we believe the mismatch(es) must occur along the 20F primer binding sequence. Although these isolates from Brazil seemed more related to genomovar I by ISR analysis, they were closer to genomovar II by

AFLP fingerprinting. Actually, the high degree of similarity between the Brazilian isolates and the channel catfish isolate ALG-00-521 suggests a common origin. To establish the geographical origin of this *Fl. columnare* strain we will need to perform a more detailed study incorporating more South American isolates as well as from Europe and Asia.

In conclusion, we confirmed the genetic instraspecific variability of *Fl. columnare* isolates from fish. We showed that both genomovars I and II are present in channel catfish from the US. We described a unique genetic group represented by four *Fl. columnare* isolates from tilapia in Brazil which appears to be related to both genomovars. We were able to further subdivide the species by analysing a less conserved ribosomic sequence (ISR). Finally, the use of AFLP® allowed us to fingerprint the species at clone level without losing the higher genetic hierarchy of genomovar division.

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